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Two factors produced in abundance by prostate cancers are prostate-specific antigen [PSA] and parathyroid hormone-related protein [PTHrP]. The latter is a major cause of osteolytic bone destruction, but the bone metastases in prostate cancer patients are usually osteoblastic. New data explain this paradox. PSA is a protease that cuts PTHrP into small fragments which cause new bone formation by binding to the endothelin receptor. Thus, PSA converts PTHrP from an osteolytic to an osteoblastic factor.

We have now shown that PTHrP 1-16, 1-20, & the PSA product 1-23, stimulate new bone formation by binding (Kd<25nM) to the endothelin A receptor. Prostate cell lines do not express both PSA [plus hK2] and PTHrP. We are reconstituting PSA+hK2 expression in a PTHrP+ cell line. We hypothesize that this will change the cell line from one that causes osteolytic metastases to one that causes osteoblastic metastases in vivo.

Neutralizing antibodies against PTHrP are currently in clinical trials. They would block both osteolytic and osteoblastic actions of PTHrP and its fragments. Our work will test whether such antibodies would be an effective treatment against skeletal metastases in prostate cancer.

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Annual Progress report, year 01: John M. Chirgwin, Ph.D.

PSA converts parathyroid hormone related protein (PTHrP) from an osteolytic to an osteoblastic factor: role in bone metastasis.

INTRODUCTION:

The skeleton is a major site of metastasis by advanced prostate cancer. Two major factors produced in abundance by such cancers are prostate-specific antigen [PSA] and parathyroid hormone-related protein [PTHrP]. The latter is a major cause of osteolytic bone destruction. However, bone metastases in prostate cancer patients are usually *osteoblastic* (characterized by net, disorganized new bone formation) rather than *osteolytic* (characterized by net bone loss). New data explain this paradox. PSA is a protease which cuts PTHrP into small fragments (Cramer et al, 1996; Iwamura et al, 1996) . Some of these fragments bind to the endothelin receptor (Schluter et al, 2001), causing a potent osteoblastic response (demonstrated for the first time here). Thus, **PSA can convert PTHrP from an *osteolytic* to an *osteoblastic* factor**. This explanation has been missed, since prostate cancer cell lines previously studied express either PSA or PTHrP, but not both. In addition, the co-expression of another protease, human kallikrein 2 [hK2] is required along with PSA [also known as hK3], since hK2 activates pro-PSA (Lovgren et al, 1997; Kumar et al, 1997).

BODY OF PROGRESS REPORT:

We proposed three hypotheses:

1) Prostate-specific antigen (PSA) was already known to cleave PTHrP to short forms, called 1-22 and 1-23, which lack osteolytic activity. Cells making active PSA have to make a related enzyme, kallikrein-2 (hK2), since it is essential for the processing of the PSA precursor. Our first hypothesis is that **hK2 cleaves PTHrP to give short forms** 1-18, 1-19, and 1-20.

2) It was very recently reported that PTHrP 1-16 is a potent ligand for the endothelin-1 type A receptor. Endothelin-1 is a potent stimulator of osteoblastic responses. We hypothesize that the **short forms of PTHrP stimulate new bone formation by binding to the endothelin receptor**.

3) Available prostate cancer cell lines do not express both PSA [plus hK2] and PTHrP. We hypothesize that **restoring PSA+hK2 expression to a PTHrP+ prostate cell line will change this cell from one that causes osteolytic metastases to one that causes osteoblastic metastases.**

Specific Aims (corresponding to Hypotheses):

1) Generate prostate cancer cell lines which make a) PTHrP alone, b) PTHrP+hK2, c) PTHrP+PSA, d) PTHrP+hK2+PSA. The production of short forms of PTHrP by these 4 cell lines will be determined. *A), b), & c) have been accomplished.*

2) Short forms of PTHrP between 1-18 and 1-23 will be tested for their ability to stimulate new bone formation. Binding to the endothelin A receptor will also be tested. *This Aim has been successfully accomplished, although more detailed receptor-binding studies remain to be carried out.*

3) The four stable cell lines made in Aim 1) will be compared in an animal model of bone metastasis. *This Aim has not yet been initiated.*

Study design (corresponding to specific aims):

1) The PC3 cell line will be used. It was isolated from a human bone metastasis and makes large amounts of intact PTHrP. It is PSA-negative. It will be transfected with DNAs expressing pro-PSA and/or pro-hK2. Pro-hK2 activates itself to mature hK2 which in turn activates PSA from its pro-form. PTHrP fragments will be purified with an antibody and their sizes determined by mass spectrometry.

2) The short fragments of PTHrP [cleaved after 18,19 or 20 by hK2 and after 22 or 23 by PSA] will be synthesized, along with positive and negative controls [1-16 and 1-34] and assayed in a neonatal mouse calvarial organ culture assay for the stimulation of new bone formation.

3) The four cell lines from Aim 1 will be inoculated into nude mice and the progression and phenotype of bone metastases determined by x-ray and histomorphometry. The type a) line causes osteolytic bone metastasis at 8 weeks in 100% of mice. The metastases should be changed to osteoblastic in the type d) line. These experiments and the corresponding third specific aim are not addressed in the first year of the proposal and are not further discussed here.

Data relating to Aim 2.

This is the most important part of the original proposal - the central idea of the Idea award, and for which no substantial preliminary data were available at the time of the review of the project. The biochemical basis of the model is shown in **Figure 1.** The sequence homology between PTHrP and endothelin-1 is highlighted. PTHrP forms 1-34 and longer act by binding to the PTH1R, a G-coupled 7-transmembrane receptor, which is modeled in **Figure 2** (Gardella & Juppner, 2001). Fragments such as 1-34 form a two-α-helix hairpin structure, which is lost when the molecule is cleaved by proteases (e.g., hK2 or PSA) at the loop between the two helices. It is also known that the first, N-terminal α-helix is weak and is substantially stabilized by the 2nd helix, which is lost upon proteolysis. The loss of stabilization by the second helix permits PTHrP fragments shorter than ~1-24 to adopt conformations able to bind the ET_A receptor. A model of the type A endothelin receptor, which is also a G-coupled 7-transmembrane receptor, is shown in **Figure 3** (Orry & Wallace, 2000). The region of homology between PTHrP and ET-1 is boxed. and "3F5" in **Figure**

1 indicates the epitope of PTHrP recognized by a monoclonal antibody able to neutralize PTHrP in vivo (Kakonen et al, 2002). The C-termini of the aligned peptides are aromatic residues in both, which could enhance binding of PSA-cleaved fragments to the ET_A receptor. These are not new data, but they demonstrate how PTHrP 1-34 and longer can activate the PTH1R without effects of the ET_A receptor, while shorter fragments have the converse actions (although PTHrP1-24 and shorter are competitors of binding to PTH1R). This information was not provided in the original proposal.

Also not available at the time of the original submission were the definitive data demonstrating that activation of the ET_A receptor was responsible for osteoblastic metastases. These studies were carried out by the P.I.'s DoD-funded colleague, Theresa A. Guise, M.D. Dr Guise collaborated with investigators at Abbott Laboratories to demonstrate the efficacy of an orally active ET_A receptor-antagonist to prevent osteoblastic bone metastases in an animal model. These experiments have been highly successful and were submitted (Yin et al) to Nature Medicine this month (Dec 2002). The results strongly support our original hypothesis, that ligands of the ET_A receptor stimulate osteoblastic new bone formation.

Using the neonatal mouse calvarial assay described in the original proposal (Traianedes et al, 1998), we have convincingly demonstrated that three fragments of PTHrP, 1-16 (commercially available and traditionally used as a negative control for the pharmacological actions of 1-34), 1-20 (synthesized for this study and predicted by us to be produced by hK2 proteolysis), and 1-23 (published by Iwamura et al, 1996 and Cramer et al, 1996, to be produced by PSA proteolysis) are potent stimulators of new bone formation in the ex vivo organ culture. We show a set of 5 figures from these extensive experiments. In each figure the upper panel shows new bone formation determined by quantitative histomorphometry of new bone area (osteoid) in fixed, stained and decalcified calvarial sections. We found similar results from either 5 or 7 days of organ culture. The results shown are from 5 day experiments. The new bone area is expressed as mm^2 (Fig 4) or μm^2 (Figs 5-8) determined for n=4 hemicalvarial sections. In each figure the bottom panel shows the osteoblast number/section counted from the same calvaria. The osteoblast number data show less change than the area of newly formed osteoid in these assays. We nevertheless provide these data and note that the osteoblast numbers are consistent with the bone formation results. We have used a variety of positive controls for these assays. The cheapest is insulin, while ET-1 is the most physiologically relevant, but the peptide is relatively unstable in solution and thus less reliable. BMP-2 and FGF-2 also give strong positive responses in the assay. The new bone formation stimulated by these two factors in *not* inhibited by the ET_A receptor antagonist ABT627 (not shown). Thus, new bone formation caused by added factors, if it is inhibited by ABT627, must occur by ligand binding to the ET_A receptor.

Figures 4 and 5 are independent experiments showing that PTHrP 1-16 effectively stimulates new bone formation and that this effect is specifically blocked by the ET_A receptor antagonist, ABT627. PTHrP 1-16 was first observed to have unexpected biological effects by Schluter et al (1997) and to bind to the ET_A receptor by Schluter et al (2001). **Figure 6** shows a similar result with PTHrP 1-20. This peptide was synthesized by the Biomolecular Protein Core in

San Antonio and purified by HPLC, as was the PTHrP1-23 peptide. The results with PTHrP 1-23 are shown in two separate experiments in **Figures 7 and 8.** We have presently tested these peptides at concentrations only as low as 25nM, at which 1-20 and 1-23 have maximal activity. Thus, we have yet to determine the lowest effective dose of these peptides to stimulate new bone formation. We believe that in the long run it will be more efficient to characterize the relationship of the structure of PTHrP peptides to their function as ligands of the ET_A receptor by carrying out classical Scatchard binding analysis of iodinated, tyrosine-containing peptides and transfected mammalian cells expressing high copy number of recombinant human ET_A receptors. We are obtaining the reagents for these experiments from Dr. Guise and will propose these experiments in a future grant application. In the original application we proposed to assay the PTHrP peptides in mouse bone marrow culture assays for their ability to stimulate osteoclast formation. We have since found that the neonatal calvarial organ culture assay will respond to exogenous 100nM PTHrP1-34 by massive osteoclastic bone destruction (not shown). We do not have currently a histomorphometric measure of this parameter, but we clearly saw no such destruction in the experiments of Figures 4-8. We are thus confident that there is no activation of osteolysis via the PTH1R in these experiments.

Data relating to Aim 1:

As described in the original proposal, we obtained reagents for hK2 expression and ELISA assays from out collaborators in Finland (Lovgren et al, 1997). We have recloned pro-hK2 and pro-PSA cDNAs into the mammalian expression vectors pcDNA3neo and pcDNA3.1zeo. We have isolated stable single-cell-derived clones by limiting dilution of PC3 pools selected for antibiotic resistance following DNA transfection. **Figure 9** shows the standard curve for the PSA ELISA, while **Figure 10** shows the results for 5 stable clones. Since these cell lines do not express hK2, the PSA is secreted in its inactive pro- form and must be assayed for immunoreactivity. Several empty vector clones show zero PSA secretion by this assay. Our highest expressing clone, 2A6, produces 100ng/ml PSA per 10^5 cells/48hrs.

At least four hK2-producing PC3 clonal sublines have also been generated (data not shown). At the present time we do not have an authentic hK2 protein standard, so we do not have absolute quantification of hK2 secretion levels. The clones were screened by hK2 ELISA and reassayed by both the ELISA and by colorimetric enzyme assay. An empty vector clone was entirely negative in both assays, while 7 clones were positive by ELISA (arbitrary average A450 values of 0.020 to 0.20). Four of the seven were also positive for active hK2 proteolytic activity with synthetic substrate (A405 values of 0.21 to 0.67), although there was no clear correlation between ELISA values and enzymatic activity. The latter requires autoactivation of pro-hK2, which may vary from clone to clone. We also have available an enzyme assay for PSA proteolytic activity.

We have prepared columns with ~5mg/ml purified 3F5 monoclonal antibody (Kakonen et al, 2002; specific for the N-terminal residues 1-4 of PTHrP) coupled to agarose by two different chemistries, using Pierce Chemical kits according to the manufacturer's instructions. These contain the expected amounts of immobilized IgG but have not been assayed for ability to bind PTHrP fragments.

Relation to specific items in the Statement of Work.

This is summarized in a modified statement of work, which follows Figure 10. Please note that no changes to the tasks have been made. The modified S.O.W. is rather a progress report submitted as part of the transfer of this project from UTHSCSA to UVa (see next section), We are not aware of any publications from other laboratories in the past year which have any direct bearing on this project; so we have not provided an update of the background section.

INSTITUTIONAL MATTERS ARISING:

This progress report technically covers the time span of 1 December 2001 through 30 November 2002. However, the funds for this project have not been available for the work proposed during part of this period. The research was actually carried out between 1 March 2002 and 30 September 2002.

Effective 1 October 2002, the P.I. and his collaborator, Theresa A. Guise, M.D., relocated to the University of Virginia, Charlottesville. Dr. Guise is now the Gerald D.Aurbach Professor of Endocrinology. She and I share 3000 square feet of dedicated space in the new Aurbach Endocrinology Research Building, as well as excellent office, laboratory core, and vivarium space, in addition to an extremely generous set-up package.

We are both members of the UVa Cancer Center and are active participants in a UVa program project. lead by Dr. Dan Theodorescu, on prostate cancer, being submitted to the NIH/NCI on Feb 1, 2003. Our project will study the role of adrenomedullin in prostate cancer bone metastases. Another investigator in this program project is Dr. Sally Parsons, Department of Microbiology, who is investigating the cAMP-mediated neuroendocrine differentation of the LNCaP prostate cancer line. She has shown that these cells produce PTHrP, as assayed with an N-terminal-specific, single antibody assay (Cox et al, 1999; Deeble et al, 2001). The cells also make active PSA and may provide a second cell line in which to study PTHrP proteolysis by PSA and hK2 and its role in osteoblastic metastases. We are actively collaborating with the Parsons laboratory to test these possibilities. We are also funded participants in the DoD Consortium, centered at Emory University under the leadership of Dr. Jonathan Simons, "Manhattan Project for Prostate Cancer: Targeting the Lethal Phenotype." Our project studies the role of IGFBP3 and its regulation by TGFbeta in prostate cancer bone metastases. We have also been joined in Virginia by Dr. Khalid Mohammad, our junior colleague, orthopaedic surgeon, and expert in histology and quantitative histomorphometry, and our senior laboratory manager, Maryla Nieuwolna. We believe that the move to UVa substantially strengthens our ability to carry out research on prostate cancer.

Shortly before the award of this grant by the DoD, in the fall of 2001, our laboratories at the University of Texas Health Science Center were relocated to an off-site research park 25 miles from the Medical Center. Not only was this highly disruptive, but the building was unsatisfactory in terms of basic cleanliness for sterile tissue culture and animal work. Support staff, especially clerical, were

also lacking. Although we are pleased to have succeeded in keeping the work proposed on schedule under these inimical conditions, we could have accomplished substantially more in a better work environment. Under the unstable circumstances, we were unable to recruit a new postdoctoral fellow to work on the project, and in Virginia we now plan to carry out the remaining work with the skills of two able and newly recruited laboratory technicians. A final problem with the environment of the University of Texas Health Science Center in San Antonio was the management of the grant accounts. At the time of our relocation to UVa, our other colleagues in bone metastasis research were relocated to yet another building and another Department, for the 3rd time in 18 months. Each relocation has required a departmental transfer of all equipment and grant accounts. At this time (12/2002), I have still not received any accounting from UTHSCSA of the funds expended by my laboratory during the first year of this grant, or of the status of any funds unexpended and their eventual transfer to UVa. A series of emails to Departmental accountants and Grants Management in San Antonio remain unanswered.

The notice of award for this proposal has not yet (to my knowledge) been effectively transferred to the University of Virginia and activated, but we have the ability to carry out the work proposed (other than animal experimentation, which requires full protocol approval) with available start-up funds.

KEY RESEARCH ACCOMPLISHMENTS:

1) Demonstration that PTHrP 1-16, 1-20, & 1-23, but not 1-34, potently stimulate new bone formation. PTHrP 1-16, 1-20, & 1-23 did not activate osteoclastic bone resorption, while 1-34 did.

2) Demonstration that PTHrP 1-16, 1-20, & 1-23 stimulate new bone formation by specific binding to the type A endothelin receptor.

3) Generation of hK2-expressing and pro-PSA-expressing stable subclones of the human prostate cancer PC3 cell line (already PTHrP-producing).

REPORTABLE OUTCOMES: none

CONCLUSIONS:

We have successfully proven the central hypothesis of the original proposal, that **PTHrP N-terminal fragments are capable of stimulating new bone formation**. This idea was novel and speculative at the time of the original submission. Thus the basic idea of the Idea award is correct.

In the subsequent two years of this proposal we will address the technically complex question of the *physiological importance* of the central hypothesis to the situation of osteoblastic metastases

seen with prostate cancer. We have made substantial progress toward this experimental goal. We are on schedule and have not made any substantive changes in our experimental plan or statement of work.

Twenty five percent of men diagnosed with prostate cancer will die from the disease. The majority of them will have painful bone metastases; which are usually osteoblastic, and for which no effective treatments are presently available (Mundy, 2002). The work proposed has already shown that PSA can convert PTHrP into an osteoblastic factor from an osteolytic one. Both PSA and PTHrP are expressed by the majority of prostate cancers. If the conversion of PTHrP by PSA into an osteoblastic factor is physiologically important in bone metastases (which will be tested *in vivo* by the work proposed for years 2 & 3), then the short forms of PTHrP are a major new target for intervention to decrease skeletal metasases in prostate cancer. A neutralizing monoclonal antibody directed against the N-terminus of PTHrP (Guise et al, 1996) has been humanized and is currently in clinical trials for the hypercalcemia of malignancy. Such antibodies would also block both osteolytic and osteoblastic actions of PTHrP and its fragments. Since the ET_A receptor is a major mediator of intractable bone pain (Mantyh et al, 2002), blockade of PTHrP ligands for this receptor should also decrease this debilitating paraneoplastic syndrome. The work proposed will provide an experimental model to test whether such antibodies would be an effective treatment against skeletal metastases in prostate cancer.

REFERENCES:

Cox ME, Deeble PD, Lakhani S, Parsons SJ (1999). Acquisition of neuroendocrine characteristics by prostate tumor cells is reversible: implications for prostate cancer progression. *Cancer Res* **59**: 3821-3830

Cramer SD, Chen Z, Peehl DM (1996). Prostate specific antigen cleaves parathyroid hormone-related protein in the PTH-like domain: inactivation of PTHrP-stimulated cAMP accumulation in mouse osteoblasts. *J Urol* **156**: 526-533

Deeble PD, Murphy DJ, Parsons SJ, Cox ME (2001). Interleukin-6- and cyclic AMP-mediated signaling potentiates neuroendocrine differentiation of LNCaP prostate tumor cells. *Mol Cell Biol* **21**: 8471-8482

Gardella TJ, Juppner H (2001). Molecular properties of the PTH/PTHrP receptor. *Trends Endocrinol Metab* **12**: 210-217

Guise TA, Yin JJ, Taylor SD, Kumagai Y, Dallas M, Boyce BF, Yoneda T, Mundy GR (1996). Evidence for a causal role of parathyroid hormone-related protein in the pathogenesis of human breast cancer-mediated osteolysis. *J Clin Invest* **98**: 1544-1549

Iwamura M, Hellman J, Cockett AT, Lilja H, Gershagen S (1996). Alteration of the hormonal

bioactivity of parathyroid hormone-related protein (PTHrP) as a result of limited proteolysis by prostate-specific antigen. *Urology* **48**: 317-325

Kakonen SM, Selander KS, Chirgwin JM, Yin JJ, Burns S, Rankin WA, Grubbs BG, Dallas M, Cui Y, Guise TA (2002). Transforming growth factor-beta stimulates parathyroid hormone-related protein and osteolytic metastases via Smad and mitogen-activated protein kinase signaling pathways. *J Biol Chem* **277**: 24571-24578

Kumar A, Mikolajczyk SD, Goel AS, Millar LS, Saedi MS (1997). Expression of pro form of prostate-specific antigen by mammalian cells and its conversion to mature, active form by human kallikrein 2. *Cancer Res* **57**: 3111-3114.]

Lovgren J, Rajakoski K, Karp M, Lundwall A , Lilja H (1997). Activation of the zymogen form of prostate-specific antigen by human glandular kallikrein 2. *Biochem Biophys Res Commun* **238**: 549-555

Mantyh PW, Clohisy DR, Koltzenburg M, Hunt SP (2002). Molecular mechanisms of cancer pain. *Nat Rev Cancer* **2**: 201-209

Mundy GR (2002). Metastasis to bone: causes, consequences and therapeutic opportunities. *Nat Rev Cancer* **2**: 584-593

Orry AJ, Wallace BA (200). Modeling and docking the endothelin G-protein-coupled receptor. *Biophys J* **79**: 3083-3094

Schluter KD, Weber M, Piper HM (1997). Effects of PTH-rP(107-111) and PTH-rP(7-34) on adult cardiomyocytes. *J Mol Cell Cardiol* **29**:3057-3065

Schluter KD, Katzer C, Piper HM (2001). A N-terminal PTHrP peptide fragment void of a PTH/PTHrP-receptor binding domain activates cardiac ET(A) receptors. *Br J Pharmacol* **132**: 427-432

Traianedes K, Dallas MR, Garrett IR, Mundy GR, Bonewald LF (1998). 5-Lipoxygenase metabolites inhibit bone formation in vitro. *Endocrinol* **139**: 3178-3184

APPENDICES:

Figures 1-10.

Revised Statement of Work (2pp).

FIGURE 1

PTHrP ↑ New Bone Formation

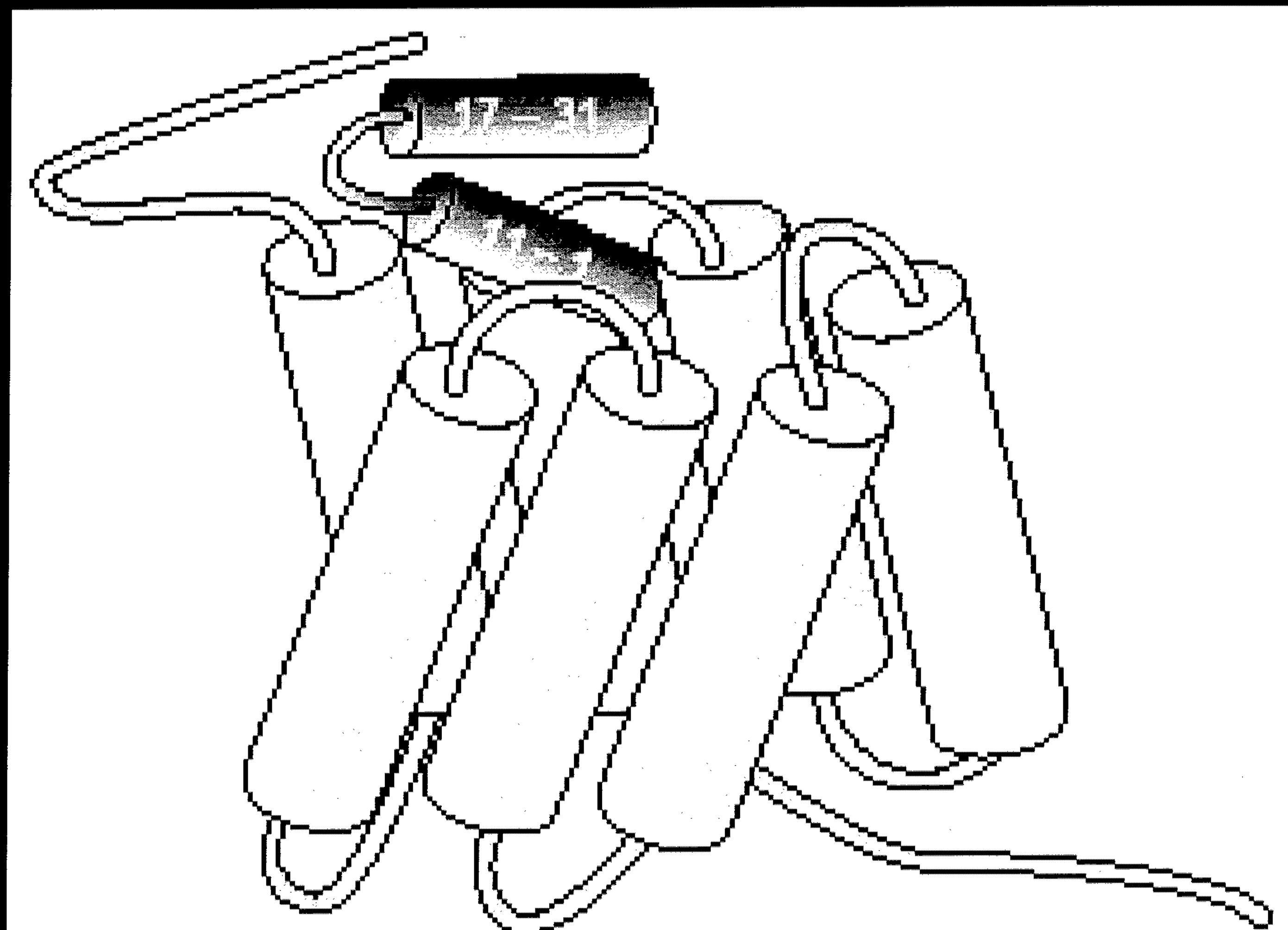
- **PTHrP 1-16 activates endothelin A receptor**
  - -Schluter et al, *Br J Pharmacol* 132:427 (2001)

```
        CSC SS L MDK ECV YFC HLD IIW    - ET-1
   1                  16  19  21 23
 AV SEH QL L HDK GKS IQD LRR RFF        - PTHrP
 3F5 epitope   ET-1 homology       ↑↑   PSA (hK3)
                                ↑ ↑ ↑   hK2 (?)
```

- **PSA cleaves PTHrP at Phenylalanines 22 & 23**
  - -Iwamura et al, *Urology* 48:317 (1996)
  - -Cramer et al, *J Urol* 156:526 (1996)

FIGURE 2

PTHrP bound to PTH1R as Two-Helix Hairpin



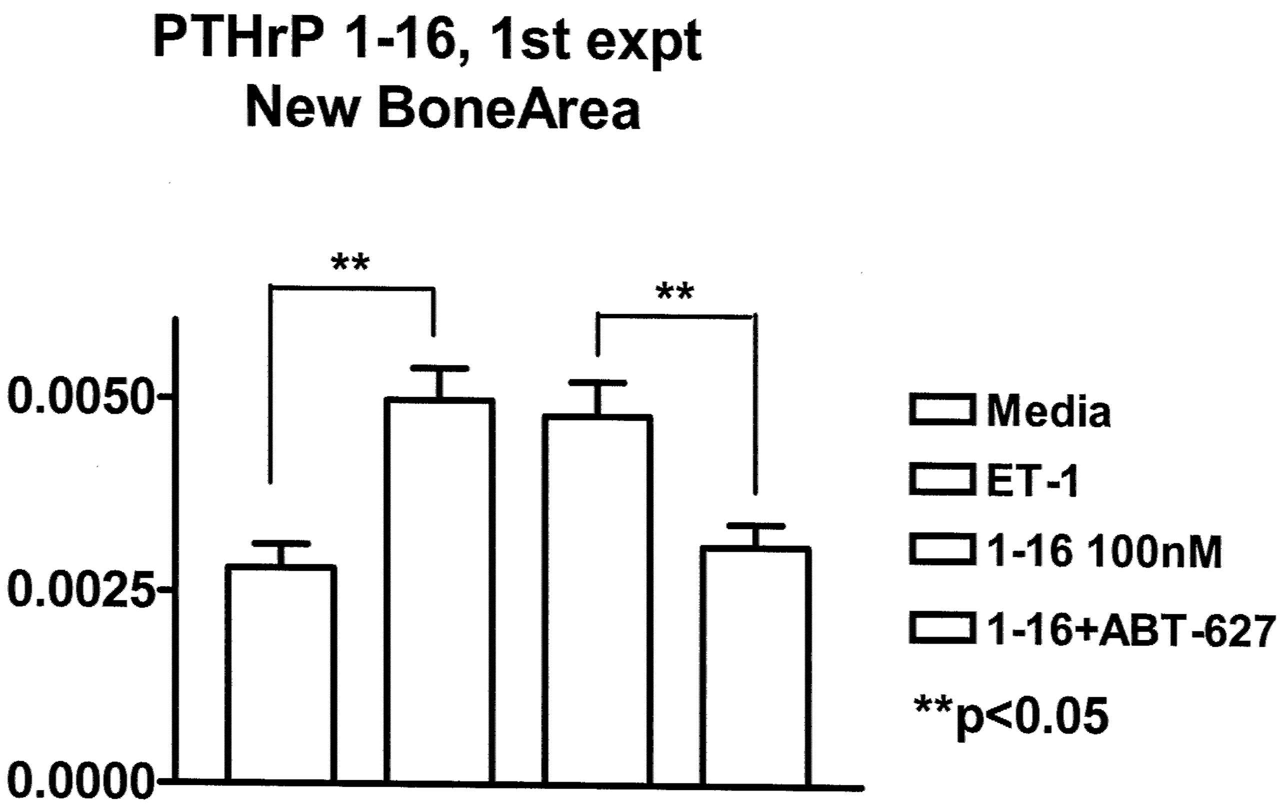
PTH model from Gardella & Juppner, *Trends Endo Metab* 12:210 (2001)

FIGURE 3

Model of ET-1 (in blue) Bound to ET_A Receptor

from Orry & Wallace, *Biophys J* 79:3083 ([illegible])

FIGURE 4


PTHrP 1-16, 1st expt
New BoneArea
**
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0.0025
0.0000
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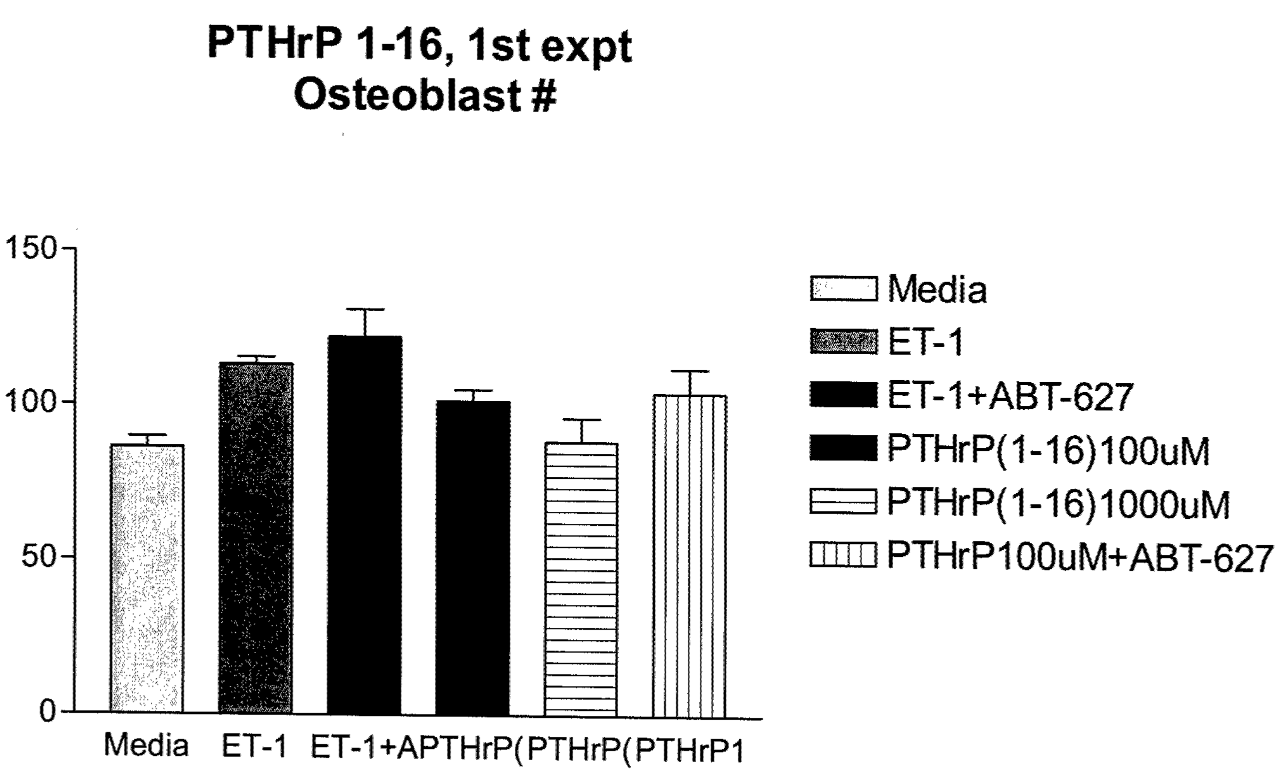
ET-1
1-16 100nM
1-16+ABT-627
**p<0.05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PTHrP1
Media
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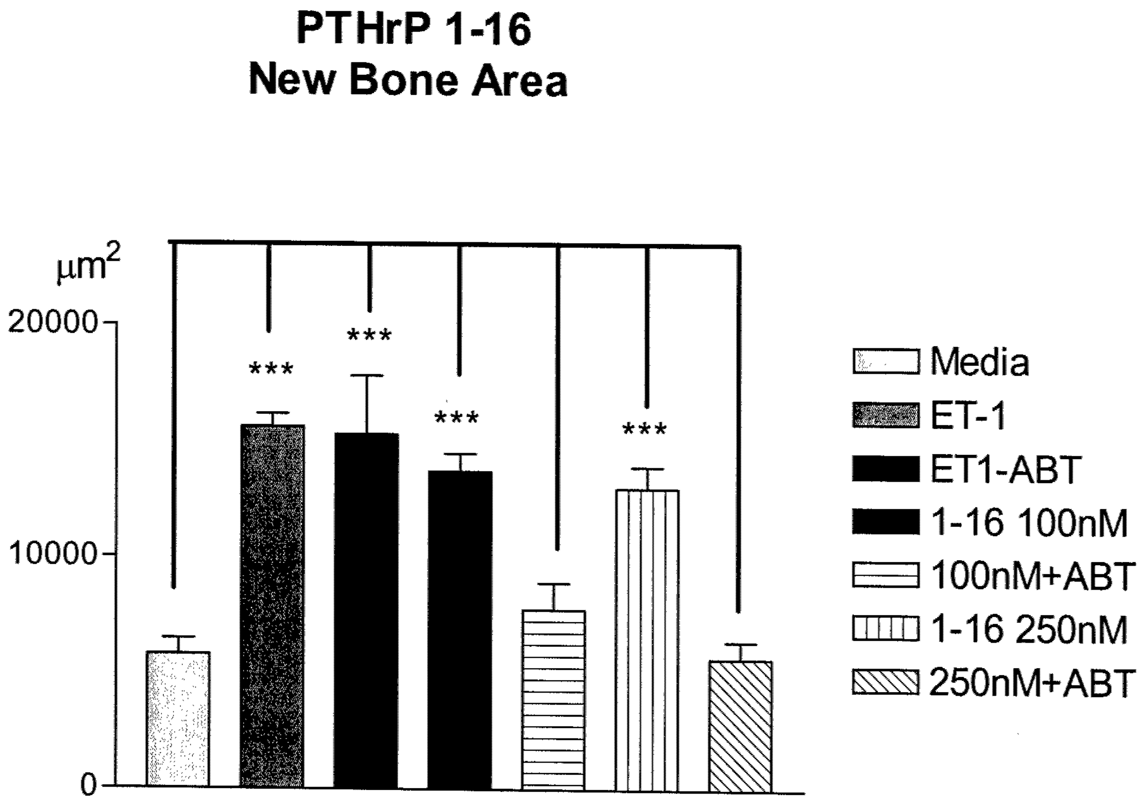
FIGURE 5


PTHrP 1-16
New Bone Area
μm2
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250nM+ABT

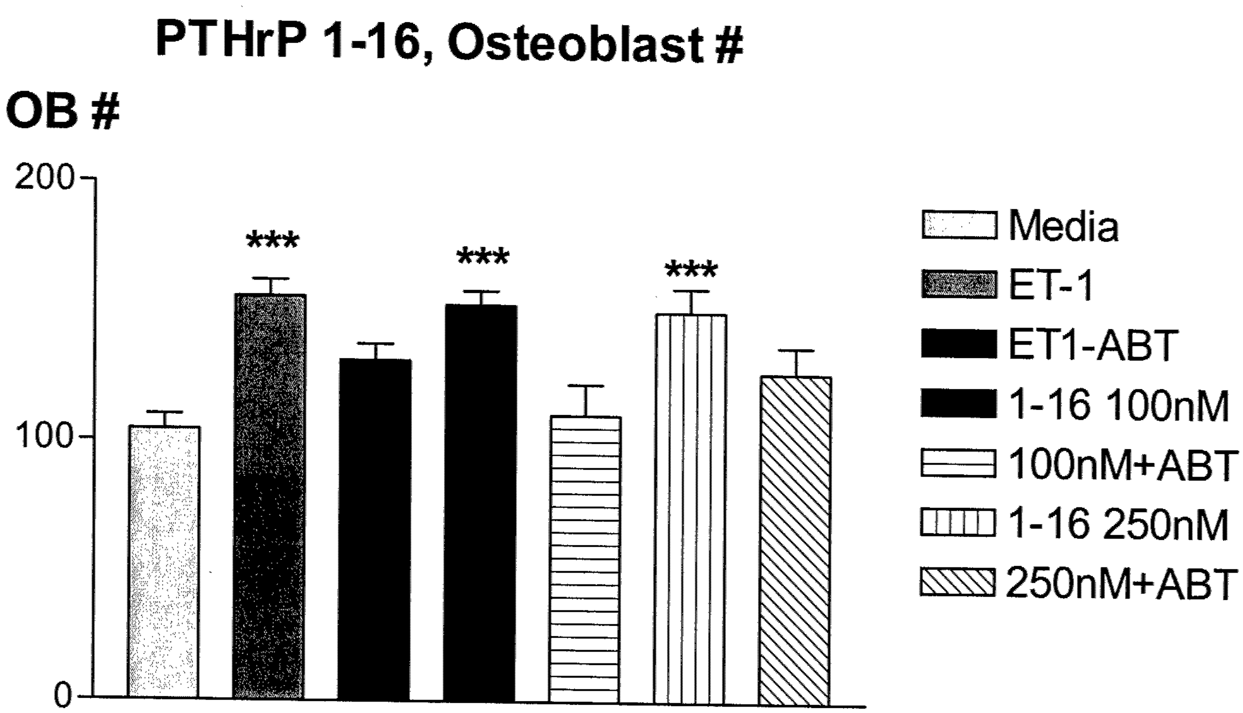
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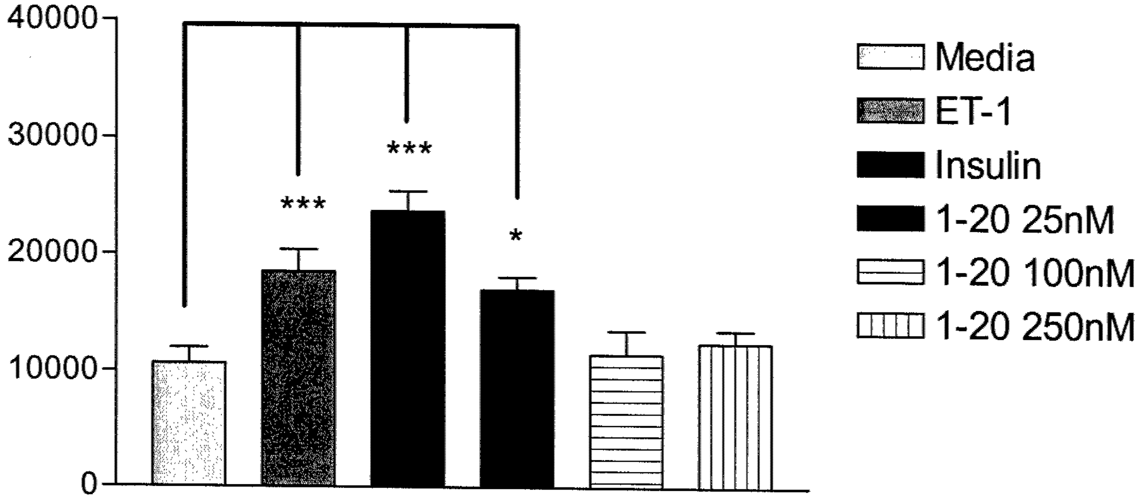
FIGURE 6

PTHrP 1-20
New Bone Area



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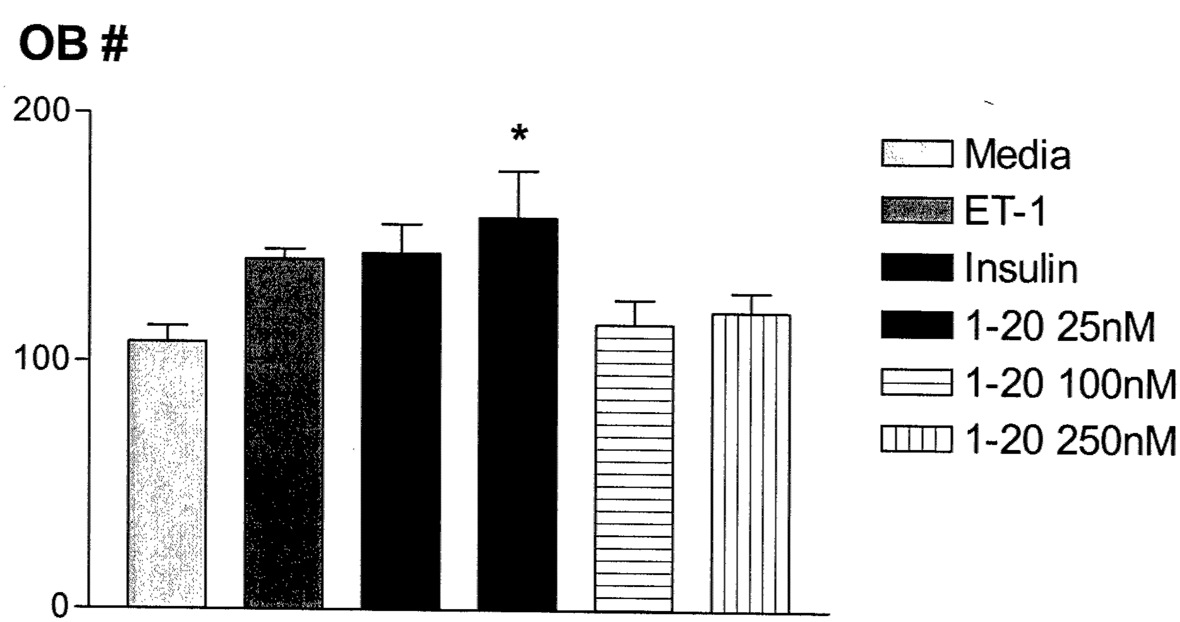

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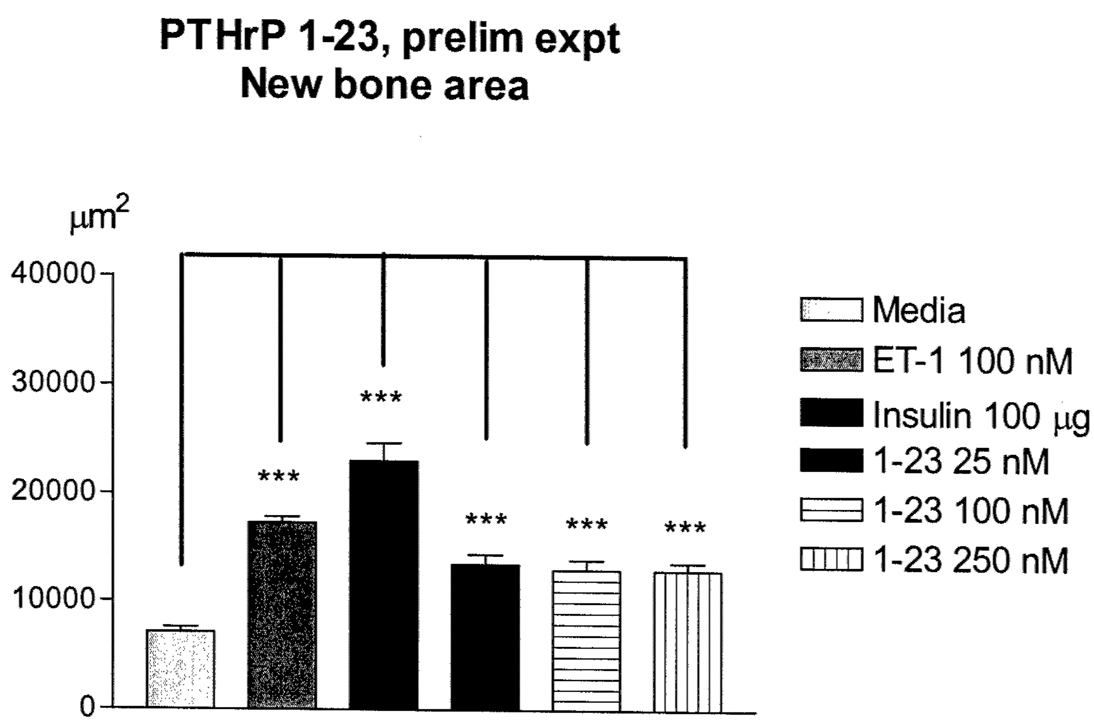
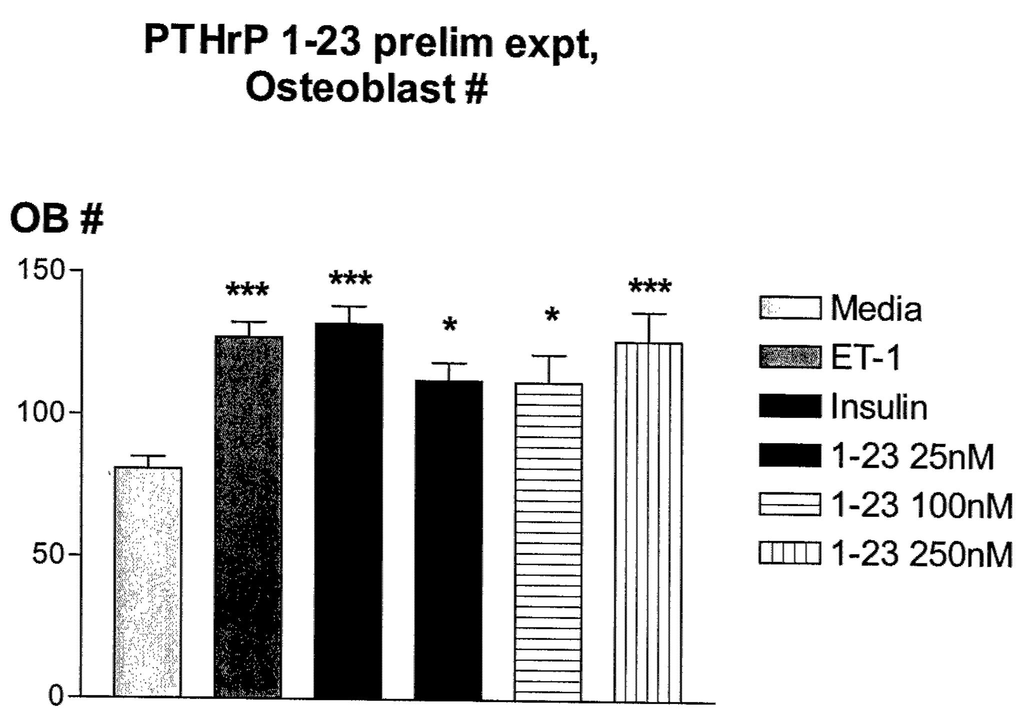


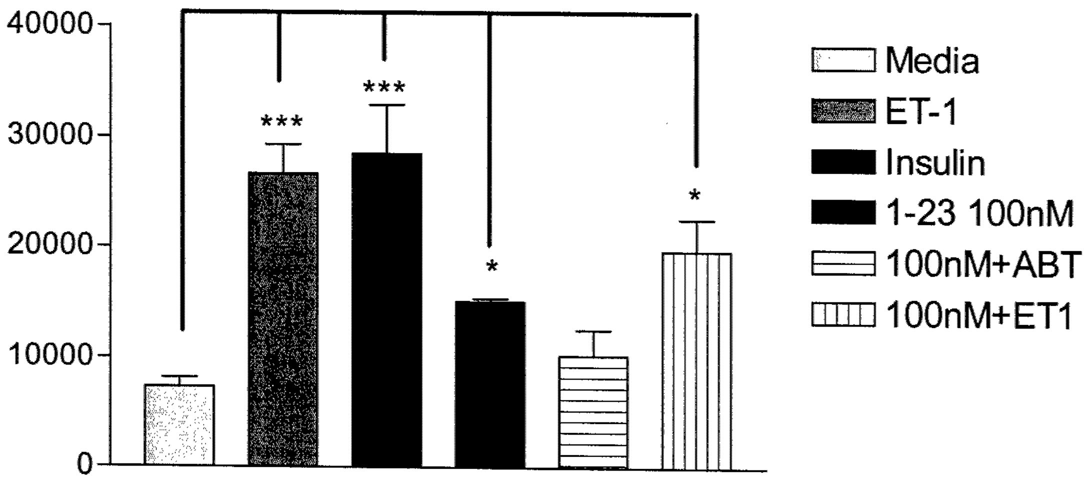
PTHrP 1-23, prelim expt
New bone area
μm²
40000
30000
20000
10000
0
***
***
***
***
***
Media
ET-1 100 nM
Insulin 100 μg
1-23 25 nM
1-23 100 nM
1-23 250 nM



PTHrP 1-23 prelim expt,
Osteoblast #
OB #
150
100
50
0
***
***
*
*
***
Media
ET-1
Insulin
1-23 25nM
1-23 100nM
1-23 250nM

FIGURE 8

PTHrP 1-23
New Bone Area



PTHrP 1-23, Osteoblast #

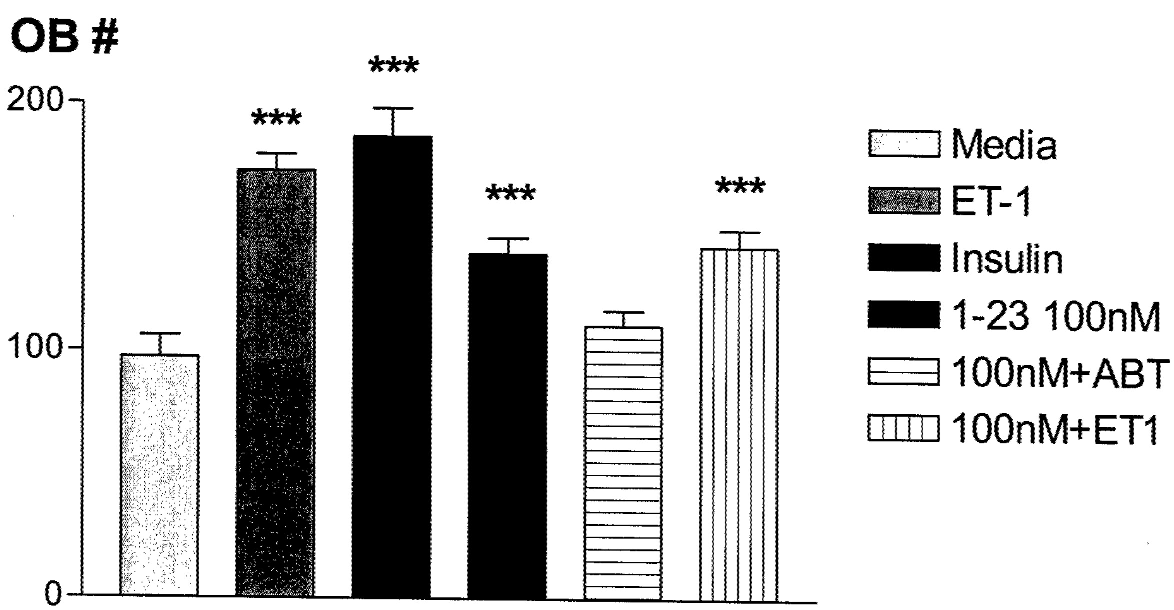


FIGURE 9

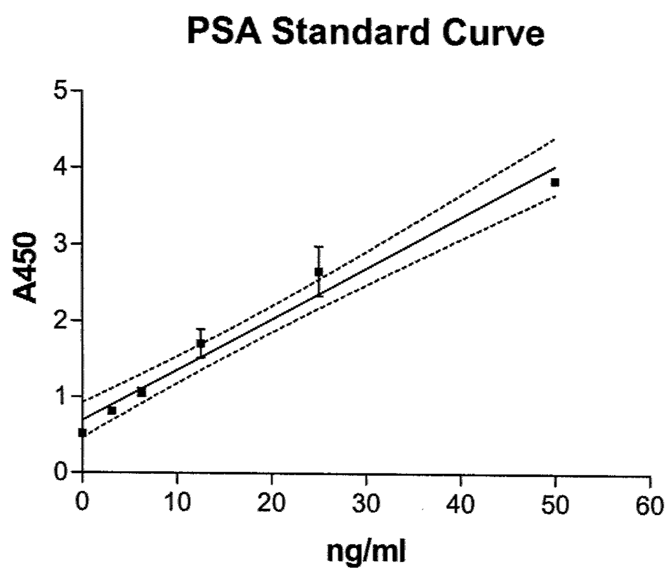
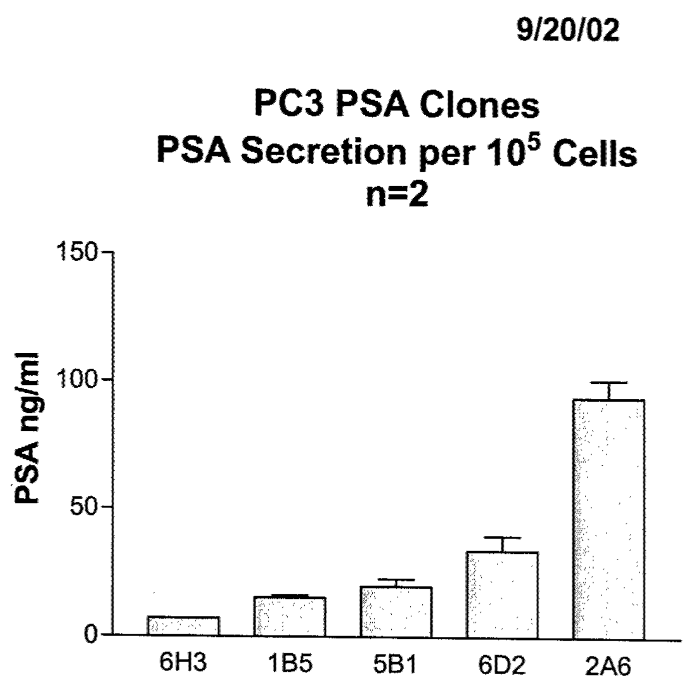


FIGURE 10


9/20/02
PC3 PSA Clones
PSA Secretion per 10^5 Cells
n=2
PSA ng/ml
150
100
50
0
6H3
1B5
5B1
6D2
2A6

STATEMENT OF WORK:

For Specific Aim 1):

Task 1: Reclone prohK2 & proPSA cDNAs into pcDNA vectors, test DNAs by transient transfections into 293 cells and assay as in task 4, below (months 1-3).

Task 2: Isolate stable single cell lines with DNAs from task 1 (months 4-12).

Task 3: Isolate stable double (hK2+PSA) cell line with DNAs from task 1 and hK2 stable clone from task 2 (months 13-24).

Task 4: Screen clones from tasks 2&3 for enzymatic activity and protein expression (months 6-12, 18-24)

Task 5: Immuno-affinity purify PTHrP fragments from media of cell lines from tasks 2&3 and analyze fragments by HPLC/mass spectrometry (months 13-18, 25-30).

For Specific Aim 2):

Task 6: Prepare synthetic peptides (months 1-3).

Task 7 Test synthetic peptides on osteoclast formation in marrow cultures (months 4-9)

Task 8: Test synthetic peptides on new bone formation in calvarial cultures (months 6-24).

Task 9: Perform tasks 7&8 on samples from task 5 (months 19-20, 31-32).

For Specific Aim 3):

Task 10: Carry out pilot animal experiment for PC3/ev and PC3/hK2 cell lines from Aim 1) in metastasis model with 8 mice (months 15-20).

Task 11: Carry out full animal experiment for the final four cell lines from Aim 1in metastasis model (n=12 mice/group) and intramuscular tumor growth rate model (n=6 mice/group) with 72 mice total (months 25-30).

Task 12: Carry out detailed histomorphometric analysis of the bones and soft tissues of the 8 mice from task 10 (bones only, months 21-24) and the 48 metastasis model mice (48+288 bone histology blocks) from the previous task (months 31-34).

Task 13: Perform statistical analysis of data from animal experiments and prepare manuscripts for publication (months 11-12, 23-24; 35-36).

Revisions to SOW consequent to relocation to University of Virginia effective 10/01/02:

The aims, tasks, and the schedule remain essentially unchanged from those originally proposed:

Task 1: complete

Task 2: underway and expected to be completed by 9/30/02

Task 3: to be initiated after transfer to Virginia

Task 4: assay procedures already established. Experiments to be initiated after transfer to Virginia

Task 5: affinity columns of immobilized anti-PTHrP N-terminal 3F5 monoclonal antibody already made. They are presently being tested and will be used as proposed after transfer to Virginia

Task 6: completed for PTHrP peptides 1-16, 1-20, 1-23, and 1-34

Tasks 7 & 8: completed for PTHrP peptides 1-16 and 1-34, underway for 1-20 and 1-23. Task 7 now uses an assay that combines the cultures of task 8, but is otherwise unchanged

Tasks 9-13: will be carried out according to the originally proposed schedule following transfer to Virginia

At this time [August 2002], all of the proposed tasks are fully on or ahead of schedule. We anticipate being able to keep to the original tasks and timetable despite the inevitable dislocations and delays associated with a cross-country move of laboratory and personnel.